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13. ABSTRACT *(Maximum 200 words)*

Settlement and metamorphosis of invertebrate larvae are key points in establishing and maintaining marine communities (including those on the bottoms of ships and piers). This research employed physiological and molecular methods to clarify (1) the basis in development of a model organism (the sea slug *Phestilla sibogae*) for establishing the capacity of larvae to metamorphose, (2) the receptor mechanisms for the larva's ability to detect settlement-stimulating chemical cues in the environment, (3) the genetic mechanisms in metamorphosis, and (4) the class of receptor molecules employed in chemoreception of the metamorphic cue. The receptor cells are included in a cilia-based organ on the head, and their specific vital staining with a vital stain allows photo-activation of metamorphosis. Metamorphic competence arises without last-minute genetic transcription or translation and probably involves morphogenesis and/or protein phosphorylation. Competent larvae survive without active cell division or genetic transcription or translation, and larvae can complete metamorphosis with all of these activities blocked. Chemical stimulation of metamorphosis causes changes in electrical activity in the brain, and the receptor for the metamorphic inducer probably is in the class of ligand-gated ion channels which transmit nervous signals at the synapse. Primary chemoreceptor cells on the cephalic tentacles of adult *P. sibogae* detect products from their prey corals and from polar amino acids. Current research builds on these findings.

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FINAL REPORT: ONR Grant No. N00014-91-J-1533.

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Project: Chemosensory Stimulation of Molluscan Settlement and Metamorphosis.

Overview: The initial objectives of the project were designed to test the hypothesis that settlement and metamorphosis of selected marine-invertebrate larvae are initiated by specific stimulation of peripheral sensory neurons and coordinated by the larval central nervous system. Subsequently, the project was broadened to examine primary chemosensory stimulation in adult organisms, as an opening into the general chemical-receptor systems used in these animals that could then be extended to larval forms. The plan was to explore electrophysiological responses and epidermal chemosensory neurons in larvae and attempt to define post-synaptic pathways that are activated during induction of metamorphosis. An AASERT addition to the project added examination of molecular mechanisms of metamorphic competence and activation in larvae of the major model organisms used in these studies, the common tropical-Pacific sea slug, *Phestilla sibogae*.

Approach: Several different methodologies were employed for different aspects of this research. The tropical sea slug, *Phestilla sibogae*, was employed as the major research model. Its larvae are easily and continuously grown in the P.I.'s laboratory, and adult animals (ca. 3 cm. long) are easily produced in abundance. The larvae are specifically induced to settle and metamorphose in response to soluble products from the prey coral of the adult slugs, a stoney coral *Porites compressa*. Using this induction system, larvae were examined by neurophysiological methods using extracellular suction electrodes attached to the larval brain while the larvae were "tested" with extracts of *P. compressa*, other non-prey corals and seawater (control). In later phases of the work, adult *P. sibogae* were investigated with electrophysiological methods. Preparations were made of the main chemosensory tentacles with their nerve connectives intact. The nerve endings were drawn into suction electrodes, and responses were recorded while exposing the tentacles to coral extracts and various concentrations of amino acids (the latter based on abundant published data on "olfactory sensitivity" of many marine animals, including sea slugs, to amino acids). The molecular basis of metamorphic induction in larvae of *Phestilla sibogae* was examined in several ways, including: (1) the ontogeny of the ability of larvae to metamorphose (competence) was explored by exposing developing larvae to inhibitors of mitosis and genetic transcription and translation; (2) longevity of competent larvae was explored in the presence of an enlarged set of inhibitors, and larvae of various ages were studied for cell cycle activity using an antibody to Proliferating-Cell-Nucleus Antigen (PCNA); (3) the capacity of competent larvae to complete metamorphosis with transcription and translation blocked was experimentally tested; (4) a cDNA library from competent larvae was constructed and probed with degenerate

oligonucleotide-primers for a family of genes known to encode olfactory-receptor proteins in rats; and (5) the morphological site of metamorphic-signal reception was explored by application of the vital mitochondrial stain DASPEI, coupled to attempts at photo-activation of metamorphosis.

Results: (1) When the larval central nervous system (CNS) of *Phestilla sibogae* is exposed and its electrical activity monitored by attaching a suction electrode, two major changes in CNS electrical activity were noted: large, regular spiking due to beating of swimming cilia became irregular and finally stopped; and a low-peak spiking from a central-pattern generator began and continued until metamorphosis occurred. (1) We have learned that the onset of metamorphic competence is not due to "last-minute" gene action, because larvae placed in strong inhibitors of genetic transcription or translation for 6 - 12 hr. on the day prior to the development of competence, developed competence on schedule (i.e., at the same time as "control" larvae). We thus now assume that the final achievement of the capacity of larvae to settle and metamorphose comes from another process such as nerve growth or the phosphorylation of a key protein(s). (2) Competent larvae live much longer in the presence of colchicine (a mitotic inhibitor), Actinomycin D and DRB (transcription inhibitors), or emetine (a translation inhibitor) than precompetent larvae, which die in great numbers within 24 hours of exposure to the agents; most competent larvae survive three days or longer. Furthermore, morphological examination revealed few mitotic nuclei in competent larvae, although a number of cells bind the PCNA antibody in the larval nervous system; we conclude that pre-metamorphic completion of these cell divisions is not essential. (4) Competent larvae can complete metamorphosis in the presence of inhibitors of mitosis, gene transcription and gene translation. Thus we conclude that competent larvae are completely "primed" for metamorphosis and do not require *de novo* gene action until the onset of post-metamorphic growth of the juvenile. (5) After repeated attempts, with appropriate successful controls, we were unable to locate any genes for seven-transmembrane-domain-receptor proteins in the cDNA library from competent larvae, and we conclude that this family of genes is not being expressed in these larvae. Other evidence now points to a different family of receptors -- ligand-gated ion channels -- as the more likely group for receiving and transducing external settlement signals in these and related larvae. Explorations of the physiology of olfaction in adult sea slugs showed unequivocally that the cephalic tentacles (rhinophores) are used in chemosensation and are very sensitive to water-soluble metabolites from corals to and polar amino acids. Preliminary anatomical explorations indicate that primary sensory units send long axons toward the brain and synapse with interneurons in the rhinophoral ganglia.

Significance: Larval settlement and metamorphosis are important as major elements in establishing and maintaining healthy populations of marine organisms on soft and hard bottoms. They are the primary steps in biofouling, a problem of great economic importance to the U.S. Navy. Detection and transduction of the chemical signals that induce metamorphosis in invertebrate larvae is a significant category of chemoreception (or olfaction) in marine organisms and one easily disrupted by pollutants and environmental toxicants. Our researches have significantly clarified the steps involved in the development of larval sensitivity to environmental chemicals, the way in which the signal is transduced, and the role of the larval nervous system in this process. Work on the adult nervous system clarified which set of tentacles is involved in chemoreception and the odorants to which the animals are sensitive; this research set the stage for physiological and molecular studies supported by a current ONR grant.

Publications:

Leise, E. M. and M. G. Hadfield. 1991. Activation of a larval central rhythm generator by a metamorphic inducer. Amer. Zool. 31:16 (abstr.).

Leise, E. M. and M. G. Hadfield 1991. Chemosensory response to metamorphic inducer activates a central pattern generator in a larval mollusc. Soc. Neurosci. Abstr. 17:1391.

Murphy, B. F., B. Ruthensteiner and M. G. Hadfield. 1993. Chemosensory abilities and pathways in the nudibranch gastropod *Phestilla sibogae*. Amino Acids 5(1):215-216.

Hadfield, M. G. and M. F. Strathmann. 1996. Variability, flexibility and plasticity in life histories of marine invertebrates. Oceanologica Acta 19:323-334.

Murphy, B. H. and M. G. Hadfield. (accepted for publication). Chemosensory pathways and responses of the nudibranch gastropod *Phestilla* sibogae. Comp. Biochem. Physiol A. (ms. 29 pp.).

Papers submitted and acknowledging this grant:

Todd, C. D., M. G. Hadfield and W. A. Snedden. (submitted) 'Juvenile" mating and sperm storage in the tropical corallivorous nudibranch, *Phestilla sibogae* (Bergh). Invert. Biol.

Papers in preparation acknowledging this grant:

Hadfield, M. G., M. F. Strathmann and R. R. Strathmann. (in prep). Ciliary currents of non-feeding veligers in ancient clades of gastropods. To be submitted to Invert. Biol.

Leise, E. M. and M. G. Hadfield. (in prep). Central nervous responses to metamorphic inducer in larvae of the nudibranch *Phestilla sibogae*. To be submitted J. Exp. Biol.

Hadfield, M. G. and E. Maleshkevitch. (in prep). Vital staining of a chemoreceptor and photo activation of metamorphosis in the nudibranch *Phestilla sibogae*. To be submitted to Biol. Bull.

Ruthensteiner, B. and M. G. Hadfield. (in prep.). Mitosis and gene action in larvae and metamorphosis in *Phestilla sibogae*. To be submitted to Biol. Bull.

Selected presentations:

1991. E.M. Leise gave contributed papers based on this research at the annual meetings of the American Society of Zoologists and the Society for Neurosciences.

1991, 1992, 1993. B. F. Murphy, B. McCauley and M. G. Hadfield presented progress reports on this research at three successive annual ONR Olfactory Discrimination Investigators' Meetings at

the Whitney Marine Laboratory in Florida.

1993. B. F. Murphy presented presented an oral paper on chemosensory physiology of *Phestilla sibogae* at the International Congress on Amino Acids in Vienna, Austria.

1994. M. G. Hadfield presented an invited paper at a symposium on "Biotic and abiotic interactions during larval and adult stages of marine benthic invertebrates." Nice, France. September 18-24, 1994.

1995. M. G. Hadfield presented an invtited talk at the "Symposium on Sensory Ecology and Physiology of Zooplankton." Honolulu, HI. January.

1996. B. McCauley spoke on "Cell proliferation, transcription and translation in metamorphosis of a mollusc." Western Regional Developmental Biology Conference."

1996. M. G. Hadfield gave "The D. P. Wilson Lecture: Past, Present and the Future in Research on Larval Settlement and Metamorphosis," the invited plenary/keynote address to the International Conference on Settlement and Metamorphosis of Marine Invertebrates, Plymouth, U.K., July 1996. Major results of the ONR-supported research were presented.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on Reverse Side.)

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1a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Michael G. Hadfield	N00014-91-J-1533	same		a. INTERIM ☐ b. FINAL ☐
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Univ. Hawaii, 41 Ahui St. Honolulu, HI 96813	1991-2-1			a. FROM 91-2-1 b. TO 96-6-30

SECTION I – SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

a. NAME(S) OF INVENTOR(S) (Last, First, MI)	b. TITLE OF INVENTION(S)	c. DISCLOSURE NO., PATENT APPLICATION SERIAL NO. OR PATENT NO.	d. ELECTION TO FILE PATENT APPLICATIONS				e. CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER	
			(1) United States		(2) Foreign			
			(a) Yes	(b) No	(a) Yes	(b) No	(1) Yes	(2) No
	NONE							

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) Name of Inventor (Last, First, MI)	(2) (a) Name of Inventor (Last, First, MI)	(1) Title of Invention	(2) Foreign Countries of Patent Application
(b) Name of Employer	(b) Name of Employer		
(c) Address of Employer (Include ZIP Code)	(c) Address of Employer (Include ZIP Code)		

SECTION II – SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

a. NAME OF SUBCONTRACTOR(S)	b. ADDRESS (Include ZIP Code)	c. SUBCONTRACT NO.(S)	d. DFAR "PATENT RIGHTS"		e. DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S)	f. SUBCONTRACT DATES (YYMMDD)	
			(1) Clause Number	(2) Date (YYMM)		(1) Award	(2) Estimated Completion

SECTION III – CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if ☐ Small Business or ☐ Non-Profit organization.) (X appropriate box)

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, MI)	c. I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.	
Hadfield, Michael G.		
b. TITLE: Professor of Zoology	d. SIGNATURE: Michael G Hadfield	e. DATE SIGNED: Nov. 16, 1996

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